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Production of indole-3-acetic acid and gibberellins A_1 and A_3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media

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Abstract

The characterization by capillary gas chromatography-mass spectrometry of the plant hormones indole-3-acetic acid and the gibberellins GA_1 and GA_3 from chemically-defined cultures of *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* is reported. Both bacteria are endophytic in gramineae species where they promote growth and yield. Quantification was also done by selected ion monitoring with [17,17-$^{2}H_2$]-Gibberellin A_1, [17,17-$^{2}H_2$]-Gibberellin A_3 and [$^{13}C_6$]-indole-3-acetic acid as internal standards. The results presented show the importance of studying phytohormonal production when the interrelationships between plants and microorganisms are analyzed and may help explain the beneficial effects of endophytic bacteria to the host plant, as has been demonstrated previously for *Azospirillum* spp.

1. Introduction

Plants are the primary sources of C and energy for soil microorganisms, either indirectly from root exudates and dead tissues, or directly when microorganisms are living inside the plant. Endophytic organisms have an advantage in that they can avoid competition for nutrients with other rhizospheric microbes. *Acetobacter diazotrophicus* has been characterized as a restricted endophyte from sugar cane, and in culture it requires media rich in sucrose (usually ~10%) [4]. *Herbaspirillum seropedicae* is a facultative endophytic bacterium that has been isolated from a number of gramineae species, including maize and sorghum [8]. Both microorganisms promote growth and yield of host plants [7].

Inoculation of cereals with N_2 fixing bacteria, such as *A. diazotrophicus* and *H. seropedicae*, may increase plant growth and grain yield [16]. However, the responses noted are inconsistent [1], and the experimental results cannot be explained only through N_2 fixation by the microorganism since the amount of N_2 already fixed is not enough to explain the total increase in N content in the inoculated plants [2]. Frequently, such inoculations augment development of the root system, thus promoting water absorption and mineral uptake [12]. Among the proposed mechanisms for this increase in root development is the bacterial production of phytohormonal substances, like cytokinins, auxins and gibberellins (GAs) [3, 11, 14, 19]. The GAs GA_1, GA_3, GA_5, $GA_9$and GA_{20} have been characterized by capillary gas chromatography-mass spectrometry (GC-MS) in chemically defined cultures of *Azospirillum lipoferum* [3, 18, Piccoli and Bottini unpublished]. Also, Crozier et al. [6] characterized indole-3-acetic acid (IAA) by GC-MS from cultures of *Azospirillum brasilense*.

The presence of IAA has been found in chemically-defined culture medium of *A. diazotrophicus*, although these results were based only on colorimetry without

Table 1. GA and IAA characterization from RP C_{18}-Nucleosil HPLC fractions of *H. seropedicae* and *A. diazotrophicus* cultures as assessed by GC-SIM with [17,17-$^2H_2$]-GA_1, [17,17-$^2H_2$]-GA_3, and [$^{13}C_6$]-IAA as internal standards

Source	C_{18}-HPLC fraction	Nucleosil-HPLC fraction	KRI*	Characteristic ion (% of abundance)		
A.diazotr.	9–11	16–17	2699	506(100)	491(9)	448(23)
H.seroped.	9–11	16–17	2699	506(100)	491(8)	448(21)
[$^2H_2$]GA_1	10–11	17	2699	508(100)	493(9)	450(21)
A.diazotr.	9–11	12	2723	504(100)	489(9)	445(7)
H.seroped.	9–11	12	2723	504(100)	489(9)	445(8)
[$^2H_2$]GA_3	10–11	12	2723	506(100)	491(10)	445(7)
A.diazotr.	10–11	–	–	319(28)	202(100)	–
H.seroped.	10–11	–	–	319(29)	202(100)	–
[$^{13}C_6$]-IAA	11	–	–	325(29)	208(100)	–

*Kovats' Retention Indices

characterization by more complete physico-chemical data such as that provided by GC-MS analysis [10].

The present work reports the characterization by GC-MS of IAA, GA_1 and GA_3, in cultures of two plant endophytic bacteria, *A. diazotrophicus* and *H. seropedicae*, grown on chemically-defined media.

2. Materials and methods

Bacterial cultures

Acetobacter diazotrophicus strain PAL 5 (ATCC 49037) and *Herbaspirillum seropedicae* strain Z67 (ATCC 35892), were a gift from Dr. Johanna Döbereiner, EMBRAPA, Itajaí, RJ, Brazil. The strains were first activated in Luria Broth (LB, Sigma Chem. Co.) medium, and then cultured on specific and selective media for each genus: LGIP for *Acetobacter spp.* and NFb for *Herbaspirillum spp.* The LGIP medium composition per liter was: sucrose 50 (5%), 100 (10%) or 150 (15%) g, K_2HPO_4 0.2 g, KH_2PO_4 0.6 g, $MgSO_4.7H_2O$ 0.2 g, $CaCl_2.2H_2O$ 0.02 g, $Na_2MoO_4.2H_2O$ 0.002 g, $FeCl_3.6H_2O$ 0.1 g, bromothymol blue 0.025 g, pH 5.5 [8], plus NH_4Cl 1.25 g. The NFb medium composition per liter was: malic acid 5 g, K_2HPO_4 0.5 g, $MgSO_4.7H_20$ 0.2 g, NaCl 0.1 g, KOH 4.5 g, $CaCl_2$ 0.02 g, $NaMoO_4.2H_2O$ 0.4 mg, $MnSO_4.H_2O$ 0.47 mg, H_3BO_3 0.56 mg, biotin/pyridoxal solution 1 ml; Fe EDTA 0.066 g; bromothymol blue 0.01 g; pH 5.8 [3], plus NH_4Cl 1.25 g. Each strain was cultured in 100 ml of the proper medium at 32 °C and 80 r.p.m. in an orbital shaker water bath until stationary phase; 2 days for *H. seropedicae* and 5 days for *A. diazotrophicus*, as determined by OD_{540} (biomass production) and by colony forming units (CFU). After incubation, the cultures were divided in two fractions, one of 20 ml for IAA determination, another of 80 ml for GA analysis.

GA analysis

Fifty ng each of [17,17-$^2H_2$]-GA_1, [17,17-$^2H_2$]-GA_3, [17,17-$^2H_2$]-GA_4, [17,17-$^2H_2$]-GA_5, [17,17-$^2H_2$]-GA_7, [17,17-$^2H_2$]-GA_9, [17,17-$^2H_2$]-GA_{19}, and [17,17-$^2H_2$]-GA_{20} (L.N. Mander, Australian National University, Adelaide, Australia) were added to the cultures. The cultures were then sonicated for 20 min and centrifuged at 10,000 xg for 30 min. The supernatant was acidified to pH 3.0 and partitioned 4 times with equal volumes of ethyl acetate. The ethyl acetate fraction (which would contained free GAs) was evaporated, diluted in 10% methanol and injected on C_{18} reverse phase (μ-Bondapack, 3.9 × 300 mm, Waters Associates) HPLC. Elution was done at 2 ml min^{-1}, in a KONIK 500 system (Konik Instruments) with a 10 to 73% gradient of methanol in 1% acetic acid. Thirty fractions of 4 ml were collected, evaporated *in vacuo*, diluted in 96% ethanol and bioassayed by the dwarf rice cv. Tan-ginbozu test modified by Nishijima & Katsura [15]. Fractions showing bioactivity were submitted to additional purification by Nucleosil HPLC, and bioassayed again. The bioactive fractions were pooled, dried and derivatized to their methyl ester-trimethylsilylethers (MeTMSi) by using ethereal diazomethane and 1:1 (v/v) dry pyridine:bistrimethylsylil-trifluoroacetamide plus 1% trimethylchlorosylane (Pierce Chemical Co.). After evaporation

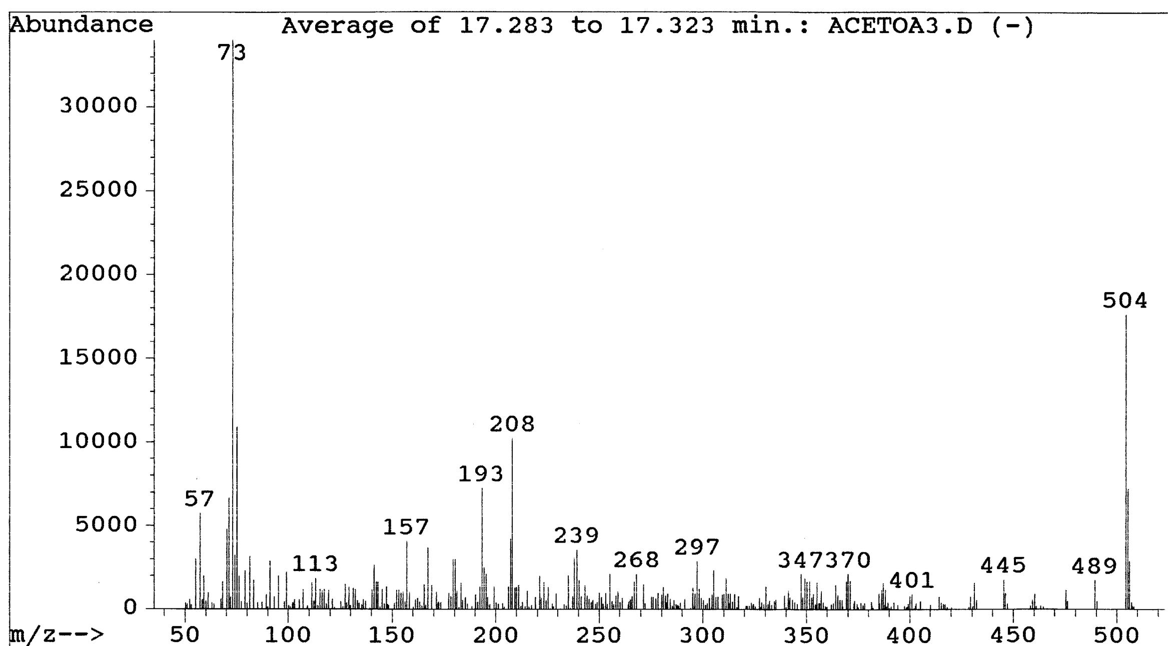

Figure 1. Full scan MS of putative GA_3 from *A. diazotrophicus* cultures.

of the reagents and redissolving in 10 μl of hexane, 1 μl of the sample was injected in the split-splitless mode in a GC-MS system consisting of a HP 5890 Series II GC connected to a HP 5972 MSD with operation and data analysis using Chemstation software running on a HP Vectra 486 computer (Hewlett Packard Co.). The GC column was a HP-5 (Hewlett Packard Co., 0.22 mm internal diameter and 25 m long, 0.1 μm film thickness) eluted with He (1 ml min^{-1}). The GC program was 100 °C to 260 °C at 20 °C min^{-1}, then 10 min at 260 °C. Three ions plus m/z 85 (for the hydrocarbon mixture) were monitored in selected ion (SIM) mode at a rate of 1.25 cycles sec^{-1}. A mixture of *n*-alkanes was co-injected in order to determine Kovats' Retention Indices (KRI's) according to Kovats [13]. The analyses were done in triplicate. Also, full scan spectra were obtained from putative bacterial GA_1 and GA_3.

IAA analysis

For IAA analysis, 20 ml of the bacterial cultures were sonicated, adjusted to pH 7, and 38 ml of isopropanol were added in order to obtain an aqueous/isopropanol ratio of 35:65. Approximately 100,000 dpm of high specific activity [^{3}H]-IAA (25 Ci mmol^{-1}) as radiotracer, and 100 ng of [$^{13}C_6$]-IAA (99atom%; stable and radioisotope labeled IAA were gifts from J.D. Cohen, Beltsville ARS-USDA, Maryland, USA) as internal standard were added to the extraction mixture for quantitative mass-spectral analysis. Following overnight isotope equilibration, the analysis of free and conjugated IAA (esters plus amides) were performed according to Chen et al. [5]. After centrifugation and evaporation to 8 ml of final volume, samples were purified by using a HPLC Beckman System Gold coupled to an UV detector (Varian UV 50). The column was a C_{18} reverse phase (Partisphere, 110 × 5 mm, Whatman Inc.) and the samples were eluted at 1 ml min^{-1} of 20% acetonitrile/water and 1% acetic acid. Quantitative IAA analysis was on a Hewlett Packard 5890-5970 GC-MS system equipped with a 12 m Chrompack CPSil 19 capillary column (i.d. 0.25 mm, film thickness 0.25 μm). Carrier gas was He at 1 ml min^{-1}, GC injector was at 280 °C and oven temperature was increased from 50 °C to 110 °C at a rate of 30 °C min^{-1} then at a rate of 6 °C min^{-1}until 280 °C. Source temperature was 270 °C and ionizing voltage was 70 eV. Samples were silylated in 50 μl acetonitrile and 50 μl of methyl-trimethylsilyl-trifluoroacetamide plus 1% trimethylchlorosylane (Merck) 15 min at room temperature. Ions monitored were m/z 202 and 208 for the base peak (quinolinium ion) and 319 and 325 for the molecular ion of the TMSi-IAA and TMSi-[$^{13}C_6$]-IAA respectively [9]. The ratio of ions at m/z 202:208 and

Table 2. GA and IAA content (in ng ml^{-1}) of *H. seropedicae* (in NFb medium) and *A. diazotrophicus* (in LGIP medium with 10 and 15% sucrose) cultures as assayed by GC-SIM with [17,17-$^2H_2$]-GA_1, [17,17-$^2H_2$]-GA_3 and [$^{13}C_6$]-IAA as internal standards

Compound	*A. diazotrophicus* (10%)	*A. diazotrophicus* (15%)	*H. seropedicae*
GA_1	1.6	–	–
GA_3	11.6	–	12.5
IAA	32	21	7
IAA-conjugated	1.4	3.3	–
IAA total	33.4	24.3	7

m/z 319:325 were used to calculate endogenous levels of IAA and triplicate injections were used to verify the analysis.

3. Results and discussion

The GA analysis showed that in *H. seropedicae* cultures GA_3 was present as shown by SIM data (Table 1). Also a full scan mass spectrum was obtained which matched that of authentic GA_3 (Figure 1). As determined from isotope dilution calculations using the SIM data from samples containing the [17,17-$^2H_2$]-GA_3 standard, the cultures produced 12.5 ng of GA_3 per ml of culture (Table 2). GAs were only characterized in *A. diazotrophicus* cultures grown on media containing 10% sucrose and in these cultures both GA_1 and GA_3 were found by SIM (Table 1) and GA_3 by full scan MS (Figure 1). SIM isotope dilution analysis with [17,17-$^2H_2$]-GA_1 and [17,17-$^2H_2$]-GA_3 standards showed that there was 1.6 ng and 11.9 ng per ml of culture medium, respectively (Table 2) with culture conditions similar to those that favored GA production by *Azospirillum lipoferum* [3, 17].

Both *H. seropedicae* and *A. diazotrophicus* produced IAA, based on GC-SIM analysis (Table 1). With *H. seropedicae,* 7 ng IAA ml^{-1} of culture was found (Table 2). IAA was found, however, only in that fraction corresponding to the free hormone. In the case of *A. diazotrophicus*, the bacteria that were cultured with 10% sucrose produced 32 ng IAA ml^{-1}, while those cultured on 15% sucrose only had 21 ng IAA ml^{-1} of culture (Table 2). Also, in this latter case, some conjugated IAA was present. No IAA was obtained from cultures grown with 5% sucrose.

These results show that both GAs and IAA are produced by *H. seropedicae* and *A. diazotrophicus* and further show the importance of studying phytohormonal production when interrelationships between plants and endophytic microorganisms are analyzed. These results also may explain, in part, the beneficial effects of these endophytic bacteria on to the host plant as has previously been demonstrated with *Azospirillum* spp. [3, 11, 14, Lucangeli and Bottini unpublished].

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